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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/00, A61K 48/00, 47/48	A1	(11) International Publication Number: WO 97/23608
		(43) International Publication Date: 3 July 1997 (03.07.97)

(21) International Application Number: PCT/US96/20543

(22) International Filing Date: 18 December 1996 (18.12.96)

(30) Priority Data:

08/577,282	22 December 1995 (22.12.95)	US
08/767,454	16 December 1996 (16.12.96)	US

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(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: COMPOSITIONS AND METHODS FOR TARGETING GENE DELIVERY VEHICLES USING COVALENTLY BOUND TARGETING ELEMENTS

(57) Abstract

The invention described herein relates to compositions and methods for targeting gene delivery vehicles. Specifically, the invention relates to utilizing multifunctional linking agents, e.g., homobifunctional, heterobifunctional, and trifunctional linking agents, to covalently bind targeting elements to the exterior of gene delivery vehicles. Following administration of such targeted gene delivery vehicles to an animal, the targeted elements interact with a specific molecule on the surface of the target cells, after which the desired nucleic acid molecule is introduced into the target cell and expressed. The described targeting mechanism allows gene delivery vehicles to be delivered to specific target cell or tissue types with greater specificity than occurs when the gene delivery vehicle administered lacks such a covalently bound targeting element.

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COMPOSITIONS AND METHODS FOR TARGETING GENE DELIVERY VEHICLES USING COVALENTLY BOUND TARGETING ELEMENTS

Technical Field

The present invention relates generally to compositions and methods for targeting gene delivery vehicles to one or more specific cell or tissue types. Specifically, the invention concerns compositions and methods which utilize targeting elements covalently bound to the surface of a gene delivery vehicle via a multifunctional linking agent.

Background of the Invention

Although many bacterial diseases can generally be treated effectively with antibiotics, very few effective treatments or prophylactic measures presently exist for many viral, cancerous, and other nonbacterial diseases, such as genetic diseases. Traditional attempts to treat these diseases have employed the use of chemical drugs. However, such drugs often lack specificity and exhibit high overall toxicity. Consequently, various methods have been developed to treat and/or prevent viral, cancerous, and genetic diseases that previously had not been amenable to traditional therapies as well as more recent therapies such as gene therapy. For example, recombinant retroviruses which can replicate and integrate into a host cell's genome through a DNA intermediate, have been utilized to deliver one or more foreign genes into a target cell in order to evoke a therapeutic benefit.

A recombinant retroviral vector expressing HIV gp160/120 envelope proteins was shown to elicit a cytotoxic T-cell response against cells infected with HIV in Warner *et al.*, (*AIDS Res. Hum. Retro.* 7:645, 1991). In Miller *et al.*, (*Science 225*:630, 1984) and Gruber *et al.*, (*Science 230*:1057, 1985) a recombinant retroviral vector expressing the hypoxanthine phosphorelase transferase (HPRT) gene was transduced into bone marrow cells and shown to produce adequate levels of HPRT to correct the metabolic defect, known as Lesch-Nyhan syndrome, in culture. However, one difficulty with recombinant retroviruses and other gene delivery vehicles is that they are difficult to target to a selected cell type or tissue where it is desired to affect treatment.

A number of methods have been devised to target viral vectors, such as retroviral vectors, to specific cell or tissue types. For example, Neda *et al.* (*J. Biol. Chem. 266*:14143,

1991) chemically coupled lactose to viral particles in order to produce viral particles capable of targeting human hepatocytes *in vitro*. However, this method is of limited applicability and has only been shown to allow the targeting of hepatocytes in tissue culture.

Others have attempted to link antibodies (Goud, *et al.*, *Vir. 163*:251, 1988) or antibody fragments (Roux *et al.*, *PNAS 86*:9070, 1989; Etienne-Julan *et al.*, *J. of Gen. Vir. 73*:3251, 1992) to a viral particle in order to target the viral particle to a specific cell type. However, while such methods produced binding of the virus to a specific cell type, it did not result in the establishment of a proviral state (Goud, *et al., supra*) or resulted in only low levels of transduction (Roux, *et al., supra* and Etienne, *et al., supra*). Moreover, none of these references described the use of such compositions to target cells *in vivo*.

Other attempts have been made to specifically target a cell type by selecting a virus which normally infects that cell type. For example, Shimada *et al.* (*J. Clin. Invest. 88*:1043, 1991) developed an HIV gene transfer system to specifically target CD4+ T-cells. However, that system produced infectious helper virus (HIV in the above example), making it unsuitable for human use. Others have co-expressed the CD4 protein in-frame with the avian leukosis virus transmembrane protein or with the transmembrane protein of murine leukemia virus in an attempt to target HIV infected T-cells (Young, *et al., Science 250*:1421, 1990). Although the CD4 protein was presented on the surface of the virus, transduction of target T-cells was not shown.

The present invention overcomes previous difficulties of delivering and specifically targeting gene delivery vehicles, and further provides other related advantages.

Summary of the Invention

The present invention provides compositions and methods for targeting gene delivery vehicles to one or more specific cell or tissue types. It is the object of the present invention to provide a gene delivery vehicle to which a targeting element is covalently bound by a linking agent such that the targeting element is capable of interacting with a molecule present on the surface of a target cell. Within one aspect, the targeting element is covalently bound to the surface of the gene delivery vehicle by a multifunctional linking agent. In certain embodiments, the multifunctional linking agent is selected from the group consisting of a homobifunctional linking agent, a heterobifunctional linking agent and a

trifunctional linking agent. In preferred embodiments, the multifunctional linking agent may be selected from the group consisting of 4(4-N-maleimidophenyl)butyric acid hydride•HCl•1/2 dioxane (MPBH), succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), sulfosuccinimidyl (4(azidosalicylamido)hexanoate (Sulfo-NHS-LC-ASA), sulfosuccinimidyl-2-[6-(biotinamido)-2-(*p*-azidobenzamido)-hexanoamido]ethyl-1,3'-dithiopropionate (Sulfo-SBED), and disuccinimidyl suberate (DSS), N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP)), sulfo-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), and bis-diazobenzidine (BDB).

In another aspect of the invention, the targeting element is covalently bound to a gene delivery vehicle by a multifunctional linking agent further comprising a carbohydrate linking agent. Preferably, the carbohydrate of the carbohydrate linking agent is a monosaccharide, a disaccharide, or an oligosaccharide. When the carbohydrate is a monosaccharide, the monosaccharide is preferably selected from the group consisting of fructosamine, glucosamine, galactosamine, and mannosamine. When the carbohydrate is a disaccharide, the disaccharide is preferably selected from the group consisting of aminated sucrose, animated maltose, aminated trehalose, and aminated lactose. When the carbohydrate is an oligosaccharide, the oligosaccharide is preferably selected from the group consisting of N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, aminated N-acetylmuramic acid, and aminated N-acetyl-D-neuraminic acid.

Any gene delivery vehicle is suitable for uses in this invention. Preferred gene delivery vehicles include recombinant viral vectors, nucleic acids associated with liposomes, nucleic acids associated with polycations, modified bacteriophage, and bacteria. When a recombinant viral vector is utilized, preferably it is a recombinant virus derived from a virus selected from the group consisting of adenovirus, astrovirus, coronavirus, hepadnevirus, orthomyxovirus, papovavirus, paramyxovirus, parvovirus, picornavirus, and poxvirus. Most preferably, the recombinant viral vector is a togavirus or a retrovirus. In particular, when the togavirus is an alphavirus, the alphavirus is preferably selected from the group consisting of Sindbis virus, Semliki Forest virus, Middleberg virus, Ross River virus, and Venezuelan equine encephalitis virus. When the recombinant viral vector is a retrovirus, the retrovirus is preferably selected from the group consisting of avian leukosis

virus, bovine leukemia virus, murine leukemia virus, mink-cell focus-inducing virus, murine sarcoma virus, reticuloendotheliosis virus, gibbon ape leukemia virus, Mason-Pfizer monkey virus, rous sarcoma virus, baboon endogenous virus, endogenous feline retrovirus (*e.g.*, RD114), and mouse or rat gL30 sequences used as a retroviral vector. When the retrovirus is a murine leukemia retrovirus, the virus is preferably selected from the group consisting of Abelson, Friend, Graffi, Kristen, Rauscher, and Moloney leukemia retrovirus, with the latter being particularly preferred. It is preferable that the recombinant viral vector is replication defective. Within a preferred embodiment of the invention, the gene delivery vehicle further comprises a fusagenic protein. Particularly preferred fusagenic proteins include those selected from the group consisting of ecotropic murine retrovirus envelope proteins, herpes simplex virus gH fusagenic proteins, herpes simplex virus gL fusagenic proteins, Epstein-Barr fusagenic proteins, measles virus fusagenic proteins, and malarial sporozoite fusagenic proteins.

Within the context of the invention, suitable targeting elements include proteins, peptides, carbohydrates, and small molecules which specifically interact with a molecule, *e.g.*, a receptor, present on the surface of the cell or tissue type(s) to be targeted. In one embodiment, the targeting element employed is a protein. In preferred embodiments, the protein-based targeting element is selected from the group consisting of a receptor, a ligand, and an antibody (or antigen binding domain thereof). In particular, when the targeting element is a receptor, the receptor is preferably selected from the group consisting of CD4, CD8, CD21, and fimbriae. When the targeting element is a ligand, the ligand is preferably selected from a cytokine, lymphokine, polypeptide hormone, peptide or nonprotein molecule. Representative cytokines include IL-1 type II, IL-2b, IL-3, IL-6, IL-7, IL-8, IL-10, and IL-12. Representative lymphokines include GM-CSF, G-CSF, M-CSF, SCF, and the flk-2 ligand. Representative polypeptide hormones include FSH, GH, luteinizing hormone, MSH, erythropoietin, nerve growth factor, VEGF, UPA, and epidermal growth factor. Representative polypeptides include neuromedin, insulin, transferrin, asialoglycoprotein, lectin, and collagen and a representative nonprotein molecule is LDL. When the targeting element is a monoclonal antibody, the monoclonal antibody is preferably selected from the group consisting of 12.8, My10, HPCA-2, anti-CD8, 4D5, GFD-OA-p185-1, CC49, B72.3, ZCEO25, c-SF-25, 14Cl, and anti-H/Le^y/Le^b.

Another aspect of the invention relates to target cells transduced with a gene delivery vehicle targeted in accordance with the teachings provided herein. In one embodiment, target cells are disease associated, *e.g.*, neoplastic cells, autoimmune cells, or cells infected with a viral or bacterial pathogen. In another embodiment, targeted cells are cells which normally express, or do not express, as the case may be, a particular gene product in an appropriate physiological amount, for example, b-islet cells in the pancreas which produce insulin in non-diabetic animals, pituitary cells which express growth hormone, and hematopoietic tissue which expresses ADA.

In another aspect of the invention, pharmaceutical compositions are provided which comprise a targeted gene delivery vehicle in a pharmaceutically acceptable carrier. Preferably, the pharmaceutical composition is lyophilized, wherein the targeted gene delivery vehicle upon reconstitution is suitable for administration to animals.

In another aspect, methods for making the compositions described herein are provided. Such methods comprise mixing a gene delivery vehicle and a targeting element in the presence of a multifunctional linking agent under conditions which allow the multifunctional linking agent to become covalently bound to both the gene delivery vehicle and targeting element(s).

Still other aspects of the invention concern methods for administering a therapeutically effective amount of the targeted gene delivery vehicles. In certain embodiments, targeted gene delivery vehicles are administered to animals in order to treat disease. Other emboidments concern the prophylactic use of targeted gene delivery vehicles in animals. Representative diseases which may be treated using targeted gene delivery vehicles include diseases selected from the group consisting of an infectious disease, cancer, a genetic disease, an autoimmune disease, and a cardiovascular disease.

Detailed Description of the Invention

The present invention provides gene delivery vehicles (GDVs) to which targeting elements have been covalently bound to the exterior surface of the GDV by a multifunctional linking agent such that the GDV is be targeted to a selected cell or tissue type.

A. Gene Delivery Vehicles

A gene delivery vehicle is a composition capable of delivering a nucleic acid molecule to a eukaryotic cell. Representative examples of gene delivery vehicles include recombinant viral vectors (*e.g.*, retroviruses; *see* WO 89/09271, and alphaviruses such as Sindbis; *see* WO 95/07994), other recombinant and non-recombinant viral systems (*e.g.*, adenovirus; *see* WO 93/19191), nucleic acid molecules associated with one or more condensing agents (*see* WO 93/03709), nucleic acid molecules associated with liposomes (Wang, *et al., PNAS 84*:7851, 1987), modified bacteriophage, or bacteria. In whatever form, the GDV carries a nucleic acid molecule to be transferred to a target cell. Once introduced, the nucleic acid molecule may itself exhibit biological activity which directly effects the target cell (*e.g.*, a ribozyme, antisense RNA, *etc.*) Alternatively, the nucleic acid molecule may encode a desired substance such as a protein, (*e.g.*, an enzyme or an antibody) and/or a nucleic acid having biological activity which, once expressed, will affect the target cell. Examples of nucleic acids which themselves have biological activity include an antisense nucleic acid molecules and ribozymes.

Preferably, the GDV is a recombinant viral vector derived from a virus such as an astrovirus, coronavirus, orthomyxovirus, papovavirus, paramyxovirus, parvovirus, picornavirus, poxvirus, retrovirus, togavirus or adenovirus. In a preferred embodiment, the recombinant viral vector is a recombinant retroviral vector. Retroviral GDVs may be readily constructed from a wide variety of retroviruses, including for example, B, C, and D type retroviruses, as well as spumaviruses and lentiviruses (*see* RNA Tumor Viruses, Second Edition, Cold Spring Harbor Laboratory, 1985). Representative examples of suitable retroviruses include those discussed in RNA Tumor Viruses, *supra*, as well as a variety of xenotropic retroviruses (*e.g.*, NZB-X1, NZB-X2 and NZB_{9-1} (*see* O'Neill *et al.*, *J. Vir. 53*:100, 1985)) and polytropic retroviruses (*e.g.*, MCF and MCF-MLV (*see* Kelly *et al., J. Vir. 45*(1):291, 1983)). Such retroviruses may be readily obtained from depositories or collections such as the American Type Culture Collection (ATCC, Rockville, MD), or isolated from known sources using commonly available techniques. Numerous retroviral GDVs which may be utilized in practicing the present invention are described in U.S. Patent Nos. 5,219,740 and 4,777,127, EP 345,242 and WO 91/02805.

Particularly preferred retroviruses are derived from retroviruses which include avian leukosis virus (ATCC Nos. VR-535 and VR-247), bovine leukemia virus (VR-1315), murine leukemia virus (MLV), mink-cell focus-inducing virus (Koch *et al.*, *J. Vir. 49*:828, 1984; and Oliff *et al.*, *J. Vir. 48*:542, 1983), murine sarcoma virus (ATCC Nos. VR-844, 45010 and 45016), reticuloendotheliosis virus (ATCC Nos VR-994, VR-770 and 45011), rous sarcoma virus, Mason-Pfizer monkey virus, baboon endogenous virus, endogenous feline retrovirus (*e.g.*, RD114), and mouse or rat gL30 sequences used as a retroviral vector. Particularly preferred strains of MLV from which recombinant retroviruses can be generated include 4070A and 1504A (Hartley and Rowe, *J. Vir. 19*:19, 1976), Abelson (ATCC No. VR-999), Friend (ATCC No. VR-245), Graffi (Ru *et al.*, *J. Vir. 67*:4722, 1993; and Yantchev *Neoplasma 26*:397, 1979), Gross (ATCC No. VR-590), Kirsten (Albino *et al.*, *J. Exp. Med. 164*:1710, 1986), Harvey sarcoma virus (Manly *et al.*, *J. Vir. 62*:3540, 1988; and Albino *et al.*, *J. Exp. Med. 164*:1710, 1986) and Rauscher (ATCC No. VR-998), and Moloney MLV (ATCC No. VR-190). A particularly preferred non-mouse retrovirus is rous sarcoma virus. Preferred rous sarcoma viruses include Bratislava (Manly *et al.*, *J. Vir. 62*:3540, 1988; and Albino *et al.*, *J. Exp. Med. 164*:1710, 1986), Bryan high titer (*e.g.*, ATCC Nos. VR-334, VR-657, VR-726, VR-659, and VR-728), Bryan standard (ATCC No. VR-140), Carr-Zilber (Adgighitov *et al.*, *Neoplasma 27*:159, 1980), Engelbreth-Holm (Laurent *et al.*, *Biochem Biophys Acta* 908:241, 1987), Harris, Prague (*e.g.*, ATCC Nos. VR-772, and 45033), and Schmidt-Ruppin (*e.g.* ATCC Nos. VR-724, VR-725, VR-354).

Any of the above retroviruses may be readily utilized in order to assemble or construct retroviral GDVs given the disclosure provided herein and standard recombinant techniques (*e.g.*, Sambrook *et al, Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, 1989 and Kunkle, *PNAS 82*:488, 1985) known in the art. In addition, within certain embodiments of the invention, portions of the retroviral GDVs may be derived from different retroviruses. For example, within one embodiment of the invention, recombinant retroviral vector LTRs may be derived from a murine sarcoma virus, a tRNA binding site from a rous sarcoma virus, a packaging signal from a MLV, and an origin of second strand synthesis from an avian leukosis virus. These recombinant retroviral vectors may be used to generate transduction competent retroviral vector particles by introducing them into appropriate packaging cell lines (*see* U.S. Patent No. 5,591,624,

issued January 1, 1996). In addition recombinant retroviruses can be produced which direct the site-specific integration of the recombinant retroviral genome into specific regions of the host cell DNA. Such site-specific integration can be mediated by a chimeric integrase incorporated into the retroviral particle. *See, for example*, US96/06727, filed May 10, 1996. It is preferable that the recombinant viral vector is a replication defective recombinant virus.

Preferably, a retroviral vector construct should include a 5' LTR, a tRNA binding site, a packaging signal, a nucleic acid molecule encoding one or more genes of interest, an origin of second strand DNA synthesis, and a 3' LTR. A retroviral vector construct may also include transcriptional promoter/enhancer or locus defining element(s), or other elements which control gene expression by means such as alternate splicing, nuclear RNA export, post-translational modification of messenger, or post-transcriptional modification of protein. Optionally, a retroviral vector construct may also include one or more selectable markers that confer resistance of vector transduced or transfected cells to thymidine kinase (TK), hygromycin, phleomycin, histidinol, or dihydrofolate reductase (DHFR), as well as one or more specific restriction sites and a translation termination sequence.

Within another preferred embodiment of the invention, the GDV is derived from a togavirus. Preferred togaviruses include alphaviruses, in particular, those described in WO 95/07994 filed September 15, 1994. A representative alphavirus in Sindbis virus. Briefly, Sindbis viral vectors typically comprise a 5' sequence capable of initiating Sindbis virus transcription, a nucleotide sequence encoding Sindbis non-structural proteins, a viral junction region inactivated so as to prevent subgenomic fragment transcription, and a Sindbis RNA polymerase recognition sequence. Optionally, the viral junction region may be modified so that subgenomic fragment transcription is reduced, increased, or maintained. As will be appreciated by those in the art, corresponding regions from other alphaviruses may be used in place of those described above. Within another embodiment, the viral junction region of an alphavirus-derived GDV may comprise a first viral junction region which has been inactivated in order to prevent transcription of the subgenomic fragment and a second viral junction region which has been modified such that subgenomic fragment transcription is reduced. Within yet another embodiment, an alphavirus-derived GDV may also include a 5' promoter capable of initiating synthesis of viral RNA from cDNA and a 3' sequence which controls transcription termination.

In other embodiments, the recombinant alphaviral vectors do not encode structural proteins and the nucleic acid molecule may be located downstream from the viral junction region. In vector constructs having a second viral junction region, the nucleic acid molecule encoding the gene(s) of interest may be located downstream from the second viral junction region. In such instances, the vector construct may further comprise a polylinker located between the viral junction region and the nucleic acid molecule. Preferably, the polylinker does not contain restriction sites found in the corresponding naturally occuring alphavirus or recombinant vector backbones made therefrom.

Other recombinant togaviral vectors that may be utilized in the present invention include those derived from Semliki Forest virus (ATCC VR-67; ATCC VR-1247), Middleberg virus (ATCC VR-370), Ross River virus (ATCC VR-373; ATCC VR-1246), Venezuelan equine encephalitis virus (ATCC VR923; ATCC VR-1250; ATCC VR-1249; ATCC VR-532), and those described within U.S. patents 5,091,309, 5,217,879, and WO 92/10578. The above described Sindbis vector constructs, as well as numerous similar vector constructs, may be readily prepared essentially as described in WO95/07994.

Similarly, the recombinant viral vector may be a recombinant adenoviral vector. Such vectors may be readily prepared and utilized given the disclosure provided herein (*see* Berkner, *Biotechniques 6*:616, 1988, and Rosenfeld *et al.*, *Science 252*:431, 1991, WO 93/07283, WO 93/06223, and WO 93/07282).

Other viral vectors suitable for use in the present invention include, for example, those derived from poliovirus (Evans *et al.*, *Nature 339*:385, 1989, and Sabin *et al.*, *J. Biol. Standardization 1*:115, 1973) (ATCC VR-58); rhinovirus (Arnold *et al.*, *J. Cell. Biochem.* L401, 1990) (ATCC VR-1110); pox viruses, such as canary pox virus or vaccinia virus (Fisher-Hoch *et al.*, *PNAS 86*:317, 1989; Flexner *et al.*, *Ann. N.Y. Acad. Sci. 569*:86, 1989; Flexner *et al.*, *Vaccine 8*:17, 1990; U.S. 4,603,112 and U.S. 4,769,330; WO 89/01973) (ATCC VR-111; ATCC VR-2010); SV40 (Mulligan *et al.*, *Nature 277:*108, 1979) (ATCC VR-305), (Madzak *et al.*, *J. Gen. Vir. 73*:1533, 1992); influenza virus (Luytjes *et al.*, *Cell 59*:1107, 1989; McMicheal *et al.*, *The New England Journal of Medicine 309*:13, 1983; and Yap *et al.*, *Nature 273*:238, 1978) (ATCC VR-797); parvovirus such as adeno-associated virus (Samulski *et al.*, *J. Vir. 63*:3822, 1989, and Mendelson *et al.*, *Virology 166*:154, 1988) (ATCC VR-645); herpes simplex virus (Kit *et al.*, *Adv. Exp. Med. Biol. 215*:219, 1989)

(ATCC VR-977; ATCC VR-260); *Nature 277*: 108, 1979); human immunodeficiency virus (EPO 386,882, Buchschacher *et al.*, *J. Vir. 66*:2731, 1992); measles virus (EPO 440,219) (ATCC VR-24); A (ATCC VR-67; ATCC VR-1247), Aura (ATCC VR-368), Bebaru virus (ATCC VR-600; ATCC VR-1240), Cabassou (ATCC VR-922), Chikungunya virus (ATCC VR-64; ATCC VR-1241), Fort Morgan (ATCC VR-924), Getah virus (ATCC VR-369; ATCC VR-1243), Kyzylagach (ATCC VR-927), Mayaro (ATCC VR-66), Mucambo virus (ATCC VR-580; ATCC VR-1244), Ndumu (ATCC VR-371), Pixuna virus (ATCC VR-372; ATCC VR-1245), Tonate (ATCC VR-925), Triniti (ATCC VR-469), Una (ATCC VR-374), Whataroa (ATCC VR-926), Y-62-33 (ATCC VR-375), O'Nyong virus, Eastern encephalitis virus (ATCC VR-65; ATCC VR-1242), Western encephalitis virus (ATCC VR-70; ATCC VR-1251; ATCC VR-622; ATCC VR-1252), and coronavirus (Hamre *et al.*, *Proc. Soc. Exp. Biol. Med. 121*:190, 1966) (ATCC VR-740).

In still another embodiment, the GDV comprises a nucleic acid molecule associated with a condensing agent (*e.g.*, polycations). Polycations condense the nucleic acid molecule by masking the negatively charged phosphate backbone, permitting the molecule to fold into a more compact form.

In an alternative embodiment, the GDV is a nucleic acid molecule associated with a liposome. Liposomes are small, lipid vesicles comprised of an aqueous compartment enclosed by a lipid bilayer, typically spherical or slightly elongated structures several hundred Angstroms in diameter. Under appropriate conditions, a liposome can fuse with the plasma membrane of a cell or with the membrane of an endocytic vesicle within a cell which has internalized the liposome, thereby releasing its contents into the cytoplasm. Prior to interaction with the surface of a cell, however, the liposome membrane acts as a relatively impermeable barrier which sequesters and protects its contents, for example, from degradative enzymes in the plasma. Additionally, because a liposome is a synthetic structure, specially designed liposomes can be produced that incorporate desirable features (*see* Stryer, L., *Biochemistry*, pp236-240, 1975 (W.H. Freeman, San Francisco, CA); Szoka *et al.*, *Biochim. Biophys. Acta 600*:1, 1980; Bayer *et al.*, *Biochim. Biophys. Acta. 550*:464, 1979; Rivnay *et al.*, *Meth. Enzymol. 149*:119, 1987; Wang *et al.*, *PNAS 84*: 7851, 1987, Plant *et al.*, *Anal. Biochem. 176*:420, 1989, and U.S. Patent No. 4,762,915). Liposomes can

encapsulate a variety of nucleic acid molecules including DNA, RNA, plasmids, and vectors such those described in the present invention.

In yet another embodiment, the GDV is a modified bacteriophage which can deliver therapeutic nucleic acid molecules to eukaryotic cells. One such representative bacteriophage system (based on bacteriophage lambda) is described in co-owned U.S.S.N. 08/366,522, filed December 30, 1994. In one embodiment, the only lambda nucleotide sequences contained in the nucleic acid molecule of such a lambda-based system are two *cos* sites, one at the 5' and 3' ends of the linear DNA to be packaged, leaving up to about 50 Kb available for therapeutic gene(s) or other sequences. These and other cosmid versions of such a gene transfer system require use of specific mutant gpJ-containing *in vitro* packaging extracts to generate infectious bacteriophage particles. Also included is an origin of replication (*e.g.*, ColE1) which allows replication in bacteria and frequently a gene coding for a selectable marker. The nucleic acid molecules are cloned into the cosmid vector between the *cos* sites (*see* WO 96/21007, filed December 20, 1995).

In another embodiment, the GDV is a bacterial cell comprising a nucleic acid molecule for delivery to eukaryotic cells. For example, the bacterial cell may express and present a cytotoxic agent, such as an anti-tumor agent, on its surface or, alternatively, secrete it into the surrounding medium. Representative examples of bacterial cell GDVs include BCG (Stover, *Nature 351:*456, 1991) and Salmonella (Newton *et al.*, *Science 244*:70, 1989).

In addition to the vector systems described above, a targeted GDV according to the invention, may carry a eukaryotic layered vector initiation system or other nucleic acid expression systems. *See* WO 95/07994 for additional details in the construction of such systems.

B. Nucleic Acid Molecules

GDVs useful in the practice of this invention above may include or contain one or more nucleic acid molecules. A wide variety of nucleic acid molecules may be utilized within the context of the present invention, including, for example, those which themselves have biological activity or which encode gene products (*e.g.*, proteins, anti-sense RNAs, and ribozymes, among others). The GDV's of the invention can contain a variety of nulceic

acid sequences of therapeutic interest. See e.g., WO 91/02805, WO 95/07994, WO 96/20414 and U.S. Patent Nos. 5,399,346, 5,580,859, 5,192,553 for a description of such nucleic acid sequences.

In yet another aspect, a targeted GDV may deliver a ribozyme directly to the target cell. Alternatively, a GDV may deliver a nucleic acid molecule which encodes one or more ribozymes (Haseloff and Gerlach, *Nature 334*:585, 1989).

In other embodiments, the nucleic acid molecule encodes one or more proteins. Representative proteins which may be encoded by a nucleic acid molecule include, for example, receptors, cytotoxins, immunomodulatory factors (*e.g.*, lymphokines and cytokines), immunoreactive proteins (*e.g.*, inhibitory, immunogenic and immunosuppressive polypeptides) and replacement proteins (*e.g.*, polypeptide hormones and enzymes expressed at insufficient levels in patients' suffering from the corresponding disease). In the case of receptors, many are involved in cell growth, either by monitoring the external environment and signaling the cell to respond appropriately. Other receptors are intracellular in nature. If either the monitoring or signaling mechanisms fail, the cell will no longer respond appropriately to particular signals and may therefore exhibit uncontrolled or aberrant growth. Many receptors or receptor-like structures may function as altered cellular components, including, for example, *neu* (also referred to as the human epidermal growth factor receptor (HER) Slamon *et al.*, *Science 244*:707, 1989; Slamon *et al.*, *Cancer Cells 7*:371, 1989; Shih *et al.*, *Nature 290*:261, 1981 Schechter, *Nature 312*:513, 1984; Coussens *et al.*, *Science 230*:1132, 1985) and mutated or altered forms of the thyroid hormone receptor, the PDGF receptor, the insulin receptor, the interleukin receptors (*e.g.*, IL-1, -2, -3, etc. receptors), or the CSF receptors, such as the G-CSF, GM-CSF, or M-CSF receptors. Alterations in these and other receptors result in the production of protein(s) or receptors containing novel coding sequence(s) which may be used as a marker of tumorigenic cells. An immune response directed against these proteins may be utilized to destroy cells expressing the altered sequence(s), as described in WO 89/09271 and WO 93/10814.

Representative examples of cytotoxins that may be encoded by nucleic acid molecule carried by a targeted GDV include, for example, ricin (Lamb et al., *Eur. J. Biochem. 148*:265-270, 1985), abrin (Wood et al., *Eur. J. Biochem. 198*:723-732, 1991;

Evensen et al., *J. of Biol. Chem. 266*:6848-6852, 1991; Collins et al., *J. of Biol. Chem. 265*:8665-8669, 1990; Chen et al., *Fed. of Eur. Biochem Soc. 309*:115-118, 1992), diphtheria toxin (Tweten et al., *J. Biol. Chem. 260*:10392-10394, 1985), cholera toxin (Mekalanos et al., *Nature 306*:551-557, 1983; Sanchez & Holmgren, *PNAS 86*:481-485, 1989), gelonin (Stirpe et al., *J. Biol. Chem. 255*:6947-6953, 1980), pokeweed (Irvin, *Pharmac. Ther. 21*:371-387, 1983), antiviral protein (Barbieri et al., *Biochem. J. 203*:55-59, 1982; Irvin et al., *Arch. Biochem. & Biophys. 200*:418-425, 1980; Irvin, *Arch. Biochem. & Biophys. 169*:522-528, 1975), tritin, Shigella toxin (Calderwood et al., *PNAS 84*:4364-4368, 1987; Jackson *et al.*, *Microb. Path. 2*:147-153, 1987), and Pseudomonas exotoxin A (Carroll and Collier, *J. Biol. Chem. 262*:8707-8711, 1987).

Within other embodiments of the invention, the targeted GDV contains a nucleic acid molecule encoding a product which is not itself toxic, but when processed or modified by a protein, such as a protease specific to a viral or other pathogen, is converted into a toxic form. For example, a GDV may carry a nucleic acid molecule encoding a proprotein which becomes toxic upon processing by a viral, *e.g.*, HIV, protease. For example, an engineered inactive proprotein form of the toxic ricin or diphtheria A chain can be cleaved to the active form by arranging for a virally encoded protease to recognize and cleave the "pro" element (see WO 95/14091).

Within other embodiments of the invention, nucleic acid molecules are provided which express one or more gene products capable of activating an otherwise inactive precursor into an active inhibitor of a pathogenic agent, or a conditional toxic palliative, *i.e.*, palliatives that are toxic for the cell expressing the pathogenic condition. A wide variety of inactive precursors may be converted into active inhibitors of a pathogenic agent. For example, antiviral nucleoside analogs such as AZT or ddC are metabolized by cellular mechanisms to a nucleotide triphosphate form in order to specifically inhibit retroviral reverse transcriptase and thus inhibit viral replication (Furmam *et al.*, *Proc. Natl. Acad. Sci. USA 83*:8333-8337, 1986). GDVs which comprise a nucleic acid molecule which encodes a substance (*e.g.*, a protein) such as herpes simplex virus thymidine kinase (HSVTK), Varicella Zoster virus thymidine kinase (VZVTK), or other such "pro-drug activating enzymes" which selectively monophosphorylate certain purine arabinosides and substituted pyrimidine compounds (*e.g.*, AZT or ddC), converting them to cytotoxic or cytostatic

metabolites, are particularly useful. Similarly, such GDVs may be utilized to express a pro-drug activating enzyme in a target cell which can be later destroyed by exposure to the appropriate "pro-drug", (*e.g.*, gancyclovir, acyclovir, or any of their analogs (*e.g.*, FIAU, FIAC, DHPG)) which is then phosphorylated into its corresponding active nucleotide triphosphate form.

In a manner similar to the preceding embodiment, a nucleic acid molecule may code for a protein which performs phosphorylation, phosphoribosylation, ribosylation, or other metabolism of a purine- or pyrimidine-based drug. Such nucleic acid molecules may have no equivalent in mammalian cells, and may be derived from organisms such as a virus, bacterium, fungus, or protozoan. Representative examples include nucleic acid molecules which encode: *E. coli* guanine phosphoribosyl transferase ("gpt"), which converts thioxanthine into thioxanthine monophosphate (*see* Besnard *et al.*, *Mol. Cell. Biol. 7*:4139, 1987); alkaline phosphatase, which converts inactive phosphorylated compounds such as mitomycin phosphate and doxorubicin-phosphate to toxic dephosphorylated compounds; fungal (*e.g.*, *Fusarium oxysporum*) or bacterial cytosine deaminase, which converts 5-fluorocytosine to 5-fluorouracil (Mullen, *PNAS 89*:33, 1992); carboxypeptidase G2, which cleaves glutamic acid from para-N-bis (2-chloroethyl) aminobenzoyl glutamic acid, to create a toxic benzoic acid mustard; and Penicillin-V amidase, which converts phenoxyacetamide derivatives of doxorubicin and melphalan to toxic compounds. Conditionally lethal products (or "pro-drugs") of this type have application to many presently known purine- or pyrimidine-based anticancer drugs, which often require intracellular ribosylation or phosphorylation to become effective cytotoxic agents. The conditionally lethal gene product could also metabolize a nontoxic drug which is not a purine or pyrimidine analog to a cytotoxic form (*see* Searle *et al.*, *Brit. J. Cancer 53*:377, 1986).

Within other embodiments of the present invention, the nucleic acid molecule carried by the targeted GDV may direct the expression of one or more immunomodulatory factors. An immunomodulatory factor is one which, when expressed by one or more of the cells involved in an immune response, or which, when added exogenously to the cells, causes an immune response to be different in quality or potency from that which would have occurred in the absence of the factor. The immunomodulatory factor may also be expressed

from an endogenous gene whose expression is driven or controlled by a gene product encoded by the nucleic acid molecule. The quality or potency of a response may be measured by a variety of known assays, for example, *in vitro* assays which measure cellular proliferation (*e.g.*, ^{3}H thymidine uptake), and *in vitro* cytotoxic assays (*e.g.*, which measure ^{51}Cr release) (*see*, Warner *et al., AIDS Res. and Human Retroviruses* 7:645, 1991). Immunomodulatory factors may be active both *in vivo* and *ex vivo*. Representative examples of such immunomodulatory factors include, for example, cytokines, such as IL-1, IL-2 (Karupiah *et al., J. Immunology 144*:290, 1990; Weber *et al., J. Exp. Med. 166*:1716, 1987; Gansbacher *et al., J. Exp. Med. 172*:1217, 1990; U.S. Patent No. 4,738,927), IL-3, IL-4 (Tepper *et al., Cell 57*:503, 1989, Golumbek *et al., Science 254*:713, 1991 and U.S. Patent No. 5,017,691), IL-5, IL-6 (Brakenhof *et al., J. Immunol. 139*:4116, 1987, and WO 90/06370), IL-7 (U.S. Patent No. 4,965,195) , IL-8, IL-9, IL-10, IL-11, IL-12 (Wolf *et al., J. Immuno. 46*:3074, 1991 and Gubler *et al., PNAS 88*:4143, 1991), IL-13 (WO 94/04680), IL-14, IL-15, a-interferon (Finter *et al., Drugs 42*(5):749, 1991, Nagata *et al., Nature* 284:316, 1980; Familletti *et al., Methods in Enz. 78*:387, 1981, Twu *et al., PNAS USA 86*:2046, 1989, Faktor *et al., Oncogene 5*:867, 1990, U.S. Patent No. 4,892,743, U.S. Patent No. 4,966,843, and WO 85/02862), b-interferon (Seif *et al., J. Vir. 65*:664, 1991), g-interferons (Radford *et al., The American Society of Hepatology* 9:2008, 1991, Watanabe *et al., PNAS 86*:9456, 1989, Gansbacher *et al., Cancer Research 50*:7820, 1990, Maio *et al., Can. Immunol. Immunother. 30*:34, 1989, U.S. Patent No. 4,762,791, and U.S. Patent No. 4,727,138), G-CSF (U.S. Patent Nos. 4,999,291 and 4,810,643), GM-CSF (WO 85/04188), tumor necrosis factors (TNFs) (Jayaraman *et al., J. Immunology 144*:942, 1990), CD3 (Krissanen *et al., Immunogenetics 26*:258, 1987), CD8, ICAM-1 (Altman *et al., Nature 338*:512, 1989; Simmons *et al., Nature 331*:624, 1988), ICAM-2 (Singer *Science 255*:1671, 1992), LFA-1 (Altmann *et al., Nature 338*:521, 1989), LFA-3 (Wallner *et al., J. Exp. Med. 166*(4):923, 1987), and other proteins such as HLA Class I molecules, HLA Class II molecules, B7 (Freeman *et al., J. Immuno. 143*:2714, 1989), B7-2, b_2-microglobulin (Parnes *et al., PNAS 78*:2253, 1981), chaperones, and MHC linked transporter proteins or analogs thereof (Powis *et al., Nature 354*:528, 1991). The choice of which immunomodulatory factor(s) to employ is based upon the therapeutic effects of the factor.

Preferred immunomodulatory factors include a-interferon, g-interferon, and IL-2 (see WO 94/21794).

Nucleic acid molecules that encode the above-described products, as well as other nucleic acid molecules that are advantageous for use within the present invention, may be readily obtained from a variety of sources, including, for example, depositories such as the American Type Culture Collection, or from commercial sources such as British Bio-Technology Limited (Cowley, Oxford England). Alternatively, cDNA sequences for use with the present invention may be obtained from cells which express or contain the sequences, such as by RT PCR from isolated mRNA. Nucleic acid molecules suitable for use with the present invention may also be synthesized in whole or in part, for example, on an Applied Biosystems Inc. DNA synthesizer (*e.g.*, ABI DNA synthesizer model 392 (Foster City, CA).

C. Targeting Elements

A targeting element is a molecule that has affinity for a molecule present on the surface of a target cell. As utilized within the context of the present invention, targeting elements are considered to be "capable of interacting with a molecule present on the surface" of a selected cell type when a biological effect of the coupled targeting element may be seen in that cell type, or, when there is greater than at least about a 10-fold difference, and preferably greater than at least about a 25, 50, or 100-fold difference, between the binding of the targeting element to target cells and non-target cells. Generally, it is preferable that the targeting element interact with a molecule present on the surface of the selected cell type with a K_D of less than about 10^{-5}M, preferably less than about 10^{-6}M, more preferably less than about 10^{-7}M, and most preferably less than about 10^{-8}M (as determined by a Scatchard analysis, *see* Scatchard, *Ann. N.Y. Acad. Sci. 51*:660, 1949). Suitable targeting elements are preferably non-immunogenic, not degraded by proteolysis, and not scavenged by the immune system. Particularly preferred targeting elements should have a half-life within an animal of between about 10 minutes and about 1 week.

Representative examples of suitable targeting elements include receptors, ligands, and antibodies (or antigen binding domains thereof). Preferable receptor targeting elements include, for example, CD4 to target B-cells, CD8 to target T-cells, and CD21 to target B-

cells. Many other suitable receptors which can be used as targeting elements in accordance with the teachings provided herein are known in the art.

When the targeting element is a ligand, it is preferably selected from a cytokine, lymphokine, polypeptide hormone, polypeptide or nonprotein molecule, for example, a carbohydrate. Preferred cytokine ligands include IL-1 type II to target myeloid cells or to target the interleukin-1 receptor on T-cells (Fanslow *et al.*, *Science 248*:739, 1990), IL2b to target B and T lymphocytes and monocytes, IL-3, SCF, or the flk-2 ligand to target hematopoietic cells, IL-6 to target activated B-cells, IL-7 to target lymphoid and myeloid cells, IL-8 to target T-cells and keratinocytes, and IL-10 to target mast cells.

Preferred lymphokine ligands include for example, GM-CSF to target granulocyte and monocyte lineage cells, G-CSF to target granulocyte lineage cells, and M-CSF to target monocyte and macrophage lineage cells.

Preferred polypeptide hormones include for example, follicle stimulating hormone (FSH) to target ovaries and testes, human growth hormone (HGH) to target osteocytes and myocytes, lutenizing hormone to target ovaries and testes, melanocyte stimulating hormone to target melanocytes, erythropoietin to target bone marrow cells, nerve growth factor to target nerve growth factor receptors on neural tumors (Chao *et al.*, *Science 232*:518, 1986), vasoendothelial growth factor (VEGF) to target cells where increased vascularization occurs, and epidermal growth factor to target epidermal cells.

Preferred polypeptides include, for example, fimbriae to target CEA receptors on cancer cells, neuromedin (Conlon, *J. Neurochem. 51*:988, 1988) to target the cells of the uterus for contractile activity, insulin to target insulin receptors on cells for glucose regulation, the Fc receptor to target macrophages (Anderson and Looney, *Immun. Today 1*:264, 1987), transferrin to target transferrin receptors on tumor cells (Huebers *et al.*, *Physio. Rev. 67*:520, 1987), asialoglycoprotein to target hepatocytes, urokinase plasminogen activator (UPA) to target endothelial cells, lectins to target specific glycoproteins or glycolipids on the surface of target cells (Sharon and Lis, *Science 246*:227, 1989), collagen type I to target colon cancer (Pullam and Bodmer, *Nature 356*:529, 1992) and acetylated low density lipoproteins ("LDL") to target macrophage scavenger receptors and atherosclerotic plaques (*see* Brown *et al.*, *Ann. Rev. Biochem 52*:223, 1983) as well as other acetylated molecules which target macrophage scavenger receptors (Paulinski *et al.*, *PNAS*

86:1372, 1989). In addition, a polypeptide targeting element which has affinity for a receptor on the target cell may be selected from libraries created utilizing recombinant techniques (*see* Scott and Smith, *Science 249*:386, 1990; Devlin *et al.*, *Science 249*:404, 1990; Houghten *et al.*, *Nature 354*:84 1991; Matthews and Wells, *Science 260*:1113,1993 and Nissim *et al.*, *EMBO J. 13*(3):692, 1994). As with receptors discussed above, numerous other polypeptide ligands suitable for use in practicing the instant invention known in the art.

Preferred nonprotein molecules include for example, targeting elements selected from existing or created organic compound libraries.

As stated above the targeting element may also be an antibody directed against a surface molecule of the target cell. Preferred antibodies include 12.8 (Andrews *et al.*, *Blood 67*:842, 1986), and My10 (Civin *et al.*, *J. Immunol. 133*:157, 1984; commercially available from Becton Dickinson under the designation HPCA-2) to target the anti-CD34 antigen on stem cells, anti-CD4 antibody to target CD4+ T-cells, anti-CD8 antibodies to target CD8+ cells, the HER2/neu monoclonal antibody 4D5 (Sarup *et al.*, *Growth Regul. 1*:72, 1991) to target ovarian and breast cells, the c-erbB-2 monoclonal antibody GFD-OA-p185-1 (Alper *et al.*, *Cell Growth Differ. 1*:591, 1990) to target breast cells, the TAG72 monoclonal antibodies CC49 and B72.3 (King *et al.*, *J. Biochem. 281*:317, 1992) to target colon and breast cells, the carcinoembryonic antigen monoclonal antibody ZCE025 (Nap *et al.*, *Canc. Res. 52*:2329, 1992) to target colon carcinoma cells, monoclonal antibody c-SF-25 to target a 125kD antigen on human lung carcinoma (Takahashi *et al, Science 259*:1460, 1993); anti-14C1 antibodies to target human ovarian cancer antigen 14C1 (Gallagher *et al.*, *Br. J. Cancer 64*:35, 1991); and anti-H/Le^y/Le^b antibodies to target lung carcinoma (Masayuki *et al.*, *N. Eng. J. Med. 327*:14, 1992). In preferred embodiments, when the cells to be targeted are human and the targeted GDV is intended to be administered *in vivo*, antibodies are preferably humanized. Techniques for the production of antibodies useful in the practice of this invention are known in the art.

In addition to those described above, other targeting elements may be utilized that are capable of interacting with a molecule present on the surface of a selected cell type or when there is a greater than at least about 10-fold difference between the binding of the targeting element to the target cells and non-target cells.

D. Multifunctional Linking Agents

Multifunctional linking agents are molecules that contain at least two reactive groups separated by a spacer or "bridge." In the practice of this invention, multifunctional linking agents are used to covalently bind a targeting element to a GDV. Upon activation of the reactive groups of the multifunctional linking agent in the presence of a GDV and a targeting element, covalent bonds are formed to link the GDV and targeting element together via the multifunctional linking agent. The spacer provides the spatial distance necessary to accommodate steric considerations of the moieties to be linked. Different linking agents may be selected based on the lengths of bridges desired for the coupling.

In one method for selecting a desired multifunctional linking agent, a linking agent with a short spacer (4-8 Å) is used and the degree of linking between the GDV and the targeting element is determined. If linking is minimal or unsuccessful, a multifunctional linking agent with a longer spacer is then selected. This process may be repeated in an iterative pattern until a linking agent providing the spacing is identified.

A bifunctional linking agent that has identical reactive groups on either end of the bridge is said to be homobifunctional. Where the reactive groups are different, the bifunctional linking agent is referred to as a heterobifunctional. Examples of reactive groups include imidoesters, N-hydroxysuccinimidyl (NHS) esters, maleimides, pyridyl disulfides, carbodiimides, and arylazides, as well as others known in the art. The imidoesters and the NHS esters react with primary amines present on the GDV and targeting element, while maleimide and pyridyl disulfide react with sulfhydryl groups present on the GDV and targeting element. Carbodiimides couple carboxyl groups to primary amines present on the GDV and the targeting element. An arylazide is a photoactivatable group that forms reactive nitrene when exposed to ultraviolet or visible light at wavelengths ranging from 250-460 nm. The aryl nitrene thus formed reacts nonselectively to form a covalent bond.

In the present invention, the targeting element is covalently bound to the GDV utilizing "multifunctional linking agents", preferably bifunctional linking agents (*e.g.*, homobifunctional or heterobifunctional linking agents). In particular, a variety of multifunctional linking agents may be utilized and are available through Pierce (Rockford,

IL). Representative multifunctional linking agents include 4(4-N-maleimidophenyl)butyric acid hydride•HCl•1/2 dioxane (MPBH; a heterobifunctional non-cleavable linking agent), succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC; a heterobifunctional linker), sulfosuccinimidyl (4(azidosalicylamido)hexanoate (sulfo-NHS-LC-ASA; a photoactivatable linking agent), sulfosuccinimidyl-2-[6-(biotinamido)-2-(p-azidobenzamido)-hexanoamido]ethyl-1,3'-dithiopropionate (Sulfo-SBED; a trifunctional linking agent having biotin covalently attached to a heterobifunctional reagent comprising a hydroxysuccinimido active ester and a photoreactive aryl azide), and disuccinimidyl suberate (DSS; a homobifunctional N-hydroxysuccinimdyl ester linker). Other multifunctional linking agents that may be utilized include, for example, N-succinimidyl-3-(2-pyridyl dithio) propionate ("SPDP"; Carlson *et al., J. Biochem. 173*:723, 1978), Sulfosuccinimidyl 4-N-maleimidomethyl) cyclohexane-1-carboxylate ("SulfoSMCC"), 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide ("EDC"), and Bis-diazobenzidine ("BDB"). Methods for conjugation of a GDV to a targeting element via a multifunctional linking agent are provided in Example 5, below.

In another embodiment of the invention, the multifunctional linking agent further comprises a monosaccharide, disaccharide or an oligosaccharide wherein the carbohydrate is first covalently bound to a targeting element utilizing the linking agents described above. The modified targeting element is then covalently bound to a GDV via the carbohydrate moiety. In a preferred embodiment of this approach, a targeting element is bound to a aminated carbohydrate utilizing a multifunctional linking agent. For example, a homobifunctional linker such as DSS may be utilized to covalently bind the targeting element to the carbohydrate via amine groups present on the targeting element and the aminated carbohydrate. Alternatively, a heterobifunctional linker such as SMCC can be used to bind the carbohydrate to a sulfhydryl present on the targeting element to the amine group of the aminated carbohydrate. The modified targeting element may then be bound to the GDV. Briefly, the GDV and the modified targeting element are mixed at various pHs ranging from about 7.4 to about 8.4 and incubated, preferably overnight at about 4°C. Following incubation, the mixture is treated with sodium cyanoborohydride. The reaction mixture is dialyzed at low temperature (about 2°C to 10°C) for a sufficient time (about 1 to 48 hours) to remove cyanoborohydride and sterilized by passage through an appropriate

filter. Alternative procedures may be employed, depending on the carbohydrate and linker employed, as those in the art will appreciate.

E. GDV Production

Once the GDV has been designed, it must be produced in an amount sufficient for conjugation to a desired targeting element and for administration to an animal. If the GDV is a recombinant viral vector, it may be produced utilizing a packaging system. A variety of viral vector packaging systems are described below in which one or more essential functions of the parent virus has been deleted so that it is deficient in some function (*e.g.*, genome replication), but retains a packaging signal and the ability to express gene products from one or more nucleic acid molecules. Representative examples of viral vector packaging systems include those for retroviral vectors, alphaviral vectors and adenoviral vectors. The deleted essential function or functions are provided by packaging cells into which the vector genome can be introduced to yield producer cell lines that then make viral particles encapsidating the recombinant viral vector. In preferred embodiments, such producer cell lines produce viral vectors substantially free from contamination with replication competent virus. The vector genome is then introduced into target cells by an infection event ("transduction") but is incapable of further propagation. In any such situation, it is important to prevent the recombination of the various parts of the virus in a producer cell line to give replication competent virus genomes, or to eliminate cells in which this occurs. The expression vector may be readily assembled from any virus utilizing standard recombinant techniques (*e.g.*, Sambrook *et. al.*, *Molecular Cloning: A Laboratory Manual*, 2d ed. Cold Spring Harbor Laboratory Press, 1989). Further description of the construction of retroviral vectors is described in WO 89/09271, herein incorporated by reference.

Within one embodiment of the present invention, the GDVs are retroviral vectors. Typically, such vectors comprise a 5' LTR, a tRNA binding site, a packaging signal, one or more genes of interest, an origin of second strand DNA synthesis, and a 3' LTR, wherein the vector lacks *gag/pol* or *env* coding sequences. As utilized herein, a 5' LTR should be understood to include a 5' promoter element and sufficient LTR sequence to allow reverse transcription and integration of the DNA form of the vector. The 3' LTR includes a

polyadenylation signal and sufficient LTR sequence to allow reverse transcription and integration of the DNA form of the vector.

When such recombinant retroviral vectors are utilized, it is preferable to utilize packaging cell lines for producing viral particles wherein at least the codons of 5' terminal end of the *gag/pol* gene are modified to take advantage of the degenerate nature of the genetic code to minimize the possibility of homologous recombination between the vector and sequences in the packaging cell coding for the viral structural proteins. Additional techniques for reducing the possibility of recombination events between vectors present in a packaging cell and the recombinant retroviral genome to be packaged are provided in WO 92/05266, WO 91/02805 and WO 96/20414.

Packaging cell lines suitable for use with the above described recombinant retroviral vectors may be readily prepared using techniques known in the art, and utilized to create producer cell lines for the production of recombinant vector particles.

In a further embodiment of the invention, alphavirus packaging cell lines are provided. In particular, alphavirus packaging cell lines are provided wherein the viral structural proteins, supplied in *trans* from one or more stably integrated expression vectors, are able to encapsidate transfected, transduced, or intracellularly produced vector RNA transcripts in the cytoplasm and release infectious, packaged vector particles through the cell membrane, thus creating an alphavirus vector producing cell line. Alphavirus RNA vector molecules, capable of replicating in the cytoplasm of the packaging cell, can be produced initially utilizing, for example, an SP6 or T7 RNA polymerase system to transcribe *in vitro* a cDNA vector clone encoding the recombinant alphaviral genome which comprises the gene(s) of interest and the alphavirus non-structural proteins. Vector RNA transcripts are then transfected into the alphavirus packaging cell line such that the vector RNA replicates to high levels and is subsequently packaged by viral structural proteins, yielding infectious vector particles.

Packaging cell lines suitable for use with the above described alphaviral vector constructs may be readily prepared (*see* WO 95/07994).

Within further embodiments of the invention, adenovirus packaging cell lines are provided. Adenovirus vectors are derived from nuclear replicating viruses and may be constructed such that they are replication defective. One or more nucleic acid molecules

may be carried by adenoviral vectors for delivery to target cells (*see* Ballay *et al.*, *EMBO J.* *4*:3861, 1985, Thummel *et al.*, *J. Mol. Appl. Genetics* *1*:435, 1982 and WO 92/05266).

Within another embodiment of the invention, a targeted gene delivery vehicle may include one or more fusigenic proteins to assist in gene delivery. Representative fusagenic proteins include ecotropic murine retrovirus envelope proteins, other retrovirus envelope proteins modified to disable normal receptor recognition, fusagenic proteins from herpes simplex virus fusagenic proteins gH and gL, Epstein-Barr virus fusagenic proteins, measles virus fusagenic proteins, malarial sporozoite fusagenic proteins, and other proteins known in the art to have fusogenic properties.

F. Purification of Gene Delivery Vehicles

Once the GDVs are produced, they are preferably purified prior to conjugation to the desired targeting element. In addition, compositions comprising targeted GDVs are preferably purified again prior to administration. The techniques utilized for purification is dependent on the type of GDV to be purified. For example, there are a variety of techniques known in the art which may be used if the GDV is an enveloped recombinant viral vector, a nucleic acid or a liposome. A preferred method is described in co-owned U.S. Patent No. 5,447,859, issued September 5, 1995. The GDVs are typically purified to a level ranging from 0.25% to 25%, and preferably about 5% to 20% before conjugation.

In addition, if the GDV is a nucleic acid, there are a variety of techniques known in the art including, for example, purification by CsCl-ethidium bromide gradient, ion-exchange chromatography, gel-filtration chromatography, and differential precipitation with polyethylene glycol. Further description of the purification of nucleic acids is provided in Sambrook *et. al.*, *Molecular Cloning: A Laboratory Manual*, 2d ed. (Cold Spring Harbor Laboratory Press, 1989).

When the GDV is a liposome, a variety of purification methods known to those skilled in the art may be utilized and are described in more detail in Mannino and Gould-Fogerite (*BioTechniques* *6*:682, 1988). Briefly, preparation of liposomes typically involves admixing solutions of one or more purified phospholipids and cholesterol in organic solvents and evaporating the solvents to dryness. An aqueous buffer containing the GDVs is then added to the lipid film and the mixture is sonicated to create a fairly uniform

dispersion of liposomes. In certain embodiments, dialysis, gel filtration, or ultracentrifugation is then be used to separate unincorporated components from the intact liposomes. (Stryer, L., *Biochemistry*, pp236 1975 (W.H. Freeman, San Francisco); Szoka *et al.*, *Biochim. Biophys. Acta 600*:1, 1980; Bayer *et al.*, *Biochim. Biophys. Acta. 550*:464, 1979; Rivnay *et al.*, *Meth. Enzymol. 149*:119, 1987; Wang *et al.*, *PNAS 84*: 7851, 1987 and, Plant *et al.*, *Anal. Biochem. 176*:420, 1989.

G. GDV/Targeting Element Production

Several linking agents may be utilized to bind target elements to GDV. The methods used vary depending on the available functional groups on the exterior of the GDV and the targeting element. For example, if the both the targeting element and the GDV have primary amines available on their surface a multifunctional linking agent such as disuccinimidyl suberate (DSS, Pierce, Rockford, IL) which is a homobifunctional N-hydroxysuccinimdyl ester linking agent. If however, the GDV contains sulfhydryl functional groups on its surface and the targeting element has a primary amine available a heterobifunctional linking agent may be utilized for example succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate. If the GDV contains a carboxyl functional group and the targeting element has a sulfhydryl functional group then a heterobifunctional group such as 4(4-N-maleimidophenyl)butyric acid hydride•HCl•1/2 dioxane may be utilized. Other linking agents may be utilized include trifunctional linking agents which permit binding of a targeting element, a GDV and a third element and light activated linking agents *see* Example 4 below. Those in the art will appreciate GDV can be bound to targeting elements by a variety of other methods.

H. Purification of GDV/Targeting Element

Several methods may be utilized for the purification of the GDV/targeting element including for example molecular sieve column chromatography (e.g., Sephadex® or Sephacryl®), equilibrium centrifugation (i.e., cesium chloride gradient centrifugation), and sucrose density gradient. Briefly, in molecular sieve column chromatography the column matrix is porous. The size of the pours in the matrix permit smaller molecule to pass into the matrix while other larger molecules are excluded from the matrix. This separates the

molecules by size eluting higher molecular weight molecule more quickly then the retained smaller molecules. Both Sephadex® and Sephacryl® are molecular sieve matrices that may be utilized to purify GDV/targeting element. Equilibrium centrifugation has been utilized in the purification of bacteriophage l (*see* Sambrook et al., Cold Spring Harbor Laboratory Press, 1989). Briefly, a cesium chloride gradient is created by layering solutions of increasing density under on another and applying an solution of the GDV/targeting element on the surface of the gradient. Upon centrifugation the GDV/targeting element will move down the gradient until the density of the cesium chloride becomes too dense for the GDV/targeting element to penetrate further. The band formed at this interface contains the purified GDV/targeting element. A sucrose gradient may be utilized to separate the GDV/targeting element similar to a cesium chloride gradient (*see* Sambrook *et al.*, *supra*).

I. Formulation

Following purification of a composition comprising a targeting element linked to a GDV via a multifunctional linking agent, the preparation is preferably formulated into a pharmaceutical composition containing some or all of the following: one or more pharmaceutically acceptable carriers and/or diluents; a saccharide; a high molecular weight structural additive; a buffering component; water; and one or more amino acids. The combination of some or all of these components acts to preserve the activity of the targeted GDV upon freezing and lyophilization, or drying through evaporation. Pharmaceutically acceptable carriers or diluents according to the invention are non-toxic to recipients at the dosages and concentrations employed. Representative examples of carriers or diluents for injectable solutions include for example water, isotonic saline solutions (*i.e.*, phosphate-buffered saline or Tris-buffered saline, preferably buffered at physiological pH), mannitol, dextrose, glycerol, and ethanol, as well as polypeptides or proteins such as human serum albumin.

The saccharide provides, among other things, support in the lyophilized or dried state. Although the preferred saccharide is lactose, other saccharides may be used, such as sucrose, mannitol, glucose, trehalose, inositol, fructose, maltose or galactose. In addition, combinations of saccharides can be used, for example, lactose and mannitol, or sucrose and

mannitol. A particularly preferred concentration of lactose is 3% to 4% by weight. Preferably, the concentration of the saccharide ranges from 1% to 12% by weight.

If the GDV of the composition is a recombinant viral vector, a preferred composition comprises 10 mg/mL mannitol, 1 mg/mL HSA, 20 mM Tris, pH 7.2, and 150 mM NaCl being particularly preferred (see WO 95/10601). Such compositions are stable at -70°C for at least six months.

The pharmaceutical compositions of the invention may also additionally include factors to stimulate cell division, and hence, uptake and incorporation of the administered GDVs. Representative examples of such factors include melanocyte stimulating hormone (MSH) for melanoma, epidermal growth factor (EGF) for breast or other epithelial carcinomas, and the anesthetic bipuvocaine (or related compounds) for intramuscular injection. Particularly preferred methods and compositions for preserving recombinant viruses are described in WO 95/10601 and WO 95/07994.

In other embodiments, differentially targeting GDVs, *i.e.*, GDVs targeted to different tissues, or the same tissue by way of a different interaction, may be provided in a single composition or each targeted GDV may be administered separately to an animal. Compositions containing multiple targeted GDVs are typically administered in the same composition, but may be simultaneously administered at the same time and same site, such as via the use of a double barreled syringe or by joint formulation. A composition containing one or more different targeted GDVs may also be administered at different sites, as disclosed in WO 96/20731.

Pharmaceutical compositions according to the invention may be provided either as a liquid solution, or as a solid form (*e.g.*, lyophilized or dehydrated) which can be resuspended in a solution prior to administration. Specifically, lyophilization involves the steps of cooling the aqueous suspension below the glass transition temperature or below the eutectic point temperature of the aqueous suspension, and removing water from the cooled suspension by sublimation. *See* Phillips *et al.*, *Cryobiology 18*:414, 1981 and WO 95/10601. The resulting composition preferably contains less than 10% water by weight. Once lyophilized, the composition is stable and may be stored at or above -70°C preferably at -20°C to -25°C.

With the evaporative method, water is removed from the aqueous suspension at ambient temperature by evaporation. For example, water may be removed through spray drying (*see* EPO 520,748). Spray drying apparatus are available from a number of manufacturers (*e.g.*, Drytec, Ltd., Tonbridge, England; Lab-Plant, Ltd., Huddersield, England). Once dehydrated, the targeted GDV composition is stable and may be stored at or above -70°C preferably at -20°C to -25°C. After preparation of the composition, where the GDV is a recombinant virus, the recombinant virus will constitute about 10 ng to 1 mg of material per dose, with about 10 times this amount of material present as copurified contaminants. Preferably, the composition is administered in doses of about 0.1 to 1.0 mL of aqueous solution, which may or may not contain one or more additional pharmaceutically acceptable excipients, stabilizers, or diluents.

Following reconstitution, the compositions are typically administered *in vivo* via traditional direct routes, such as buccal/sublingual, rectal, oral, nasal, topical, (such as transdermal and ophthalmic), vaginal, pulmonary, intraarterial, intramuscular, intraperitoneal, subcutaneous, intraocular, intranasal, intravenous routes or directly into a specific tissue, such as the liver, bone marrow, or into the tumor in the case of cancer therapy.

Preferably, the composition is administered to an animal via the desired route and then the animal is tested for the desired biological response. Such testing may include immunological screening assays *e.g.*, CTL assays, antibody assays. The test(s) performed will depend on the product produced by the nucleic acid molecule introduced by the targeted GDV and the disease to be treated or prevented. On the basis of the results of such testing, the titers of the targeted GDVs to be administered may be adjusted to further enhance the desired effect(s).

The following examples are offered by way of illustration and not by way of limitation.

Example 1

COVALENT LINKAGE OF TARGETING ELEMENT TO GDVS

Linking agents are utilized to link GDVs to the selected targeting elements. These linking agents contain at least two reactive groups which, upon activation in the presence of GDV and the targeting element, form covalent bonds thereby coupling the GDV and

targeting element. When the reactive groups are identical, the linking agent is said to be homobifunctional. If the reactive groups are different, the linking agent is referred to as a heterobifunctional. Examples of reactive groups include imidoesters, N-hydroxysuccinimidyl (NHS) esters, maleimides, pyridyl disulfides, carbodiimides, and arylazides. The imidoesters and the NHS esters react with primary amines present on GDVs and targeting elements while the maleimide and pyridyl dissulfide react with sulfhydryl groups present on GDVs and targeting elements. Carbodiimides couple carboxyl groups to primary amines present on the GDV and the targeting elements. A photoactivatable arylazide is a group that is photolyzed when exposed to ultraviolet or visible light at wavelengths ranging from 250-460 nm to form a reactive nitrene. The aryl nitrene thus formed non-selectively forms a covalent bond.

In addition, the linking agent may further comprise a monosaccharide, disaccharide or an oligosaccharide, wherein the carbohydrate is covalently bound to a targeting element utilizing the linking agents described above and then the modified targeting element is covalently bound to a GDV via the carbohydrate.

A. Conjugation using 4(4-N-maleimidophenyl)butyric acid hydride•HCl•1/2 dioxane

4(4-N-maleimidophenyl)butyric acid hydride•HCl•1/2 dioxane (MPBH, Pierce, Rockford, IL) is a heterobifunctional non-cleavable linking agent containing a hydrazide group and maleimide that react with carbohydrates and sulfhydryls, respectively. This protocol provides for the conjugation of glycoproteins present on the surface of a recombinant virus, a polycation, a liposome, bacteriophage or bacterium to thiol-containing proteins of a targeting element. The recombinant virus is first conjugated to MPBH followed by conjugating to the sulfhydryl-containing targeting element. Briefly, approximately 1.0 mL of cold recombinant virus solution having an equivalent protein concentration of about 10 mg/mL is added to 0.1 mL of a cold sodium *meta*-periodate solution containing 100 mM sodium periodate in 0.1 M sodium acetate buffer, pH 5.5. The oxidation reaction is allowed to proceed for 1.0 hour in the dark at room temperature. Glycerol is added to the mixture to a final concentration of 15 mM and the reaction mixture is incubated for 5 minutes at 0°C. This mixture is then centrifuged at 1000 x g for 15 to 30 minutes using a microconcentrator. Following incubation, the reaction mixture is brought

back to its original volume with 0.1 M sodium acetate buffer, pH 5.5, and the centrifugation procedure is repeated two additional times. A 10 mg/mL solution of MPBH is added to the oxidized recombinant virus to a final concentration of 1 mM MPBH and allowed to react with agitation for 2.0 hours at room temperature. The excess MPBH is removed by centrifugation at 1000 x g for 15 to 30 minutes using a microconcentrator. The sample is then brought back to its original volume in 0.1 M sodium phosphate, 50 mM NaCl, pH 7.0. This centrifugation process is repeated twice. Following centrifugation 0.5 mL of the solution of the targeting element (5 mg/mL of the targeting element in 0.1 M sodium phosphate, 50 mM NaCl, pH 7.0 buffer) is added to the MPBH-modified recombinant virus. This reaction mixture is incubated for 2.0 hours at room temperature. The targeting element-conjugated recombinant virus may then be purified by column chromatography.

B. Conjugation using Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate

Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Pierce, Rockford, IL) linking agent consists of an N-hydroxysuccinimide (NHS)-ester and a maleimide group connected with a spacer. The NHS ester reacts with a primary amine at pH 7 to 9 and the maleimide reacts with sulfhydryl groups at pH 6.5 to 7.5. This protocol provides for the conjugation of a recombinant virus, a polycation, a liposome, bacteriophage or bacterium to a targeting element via sulfhydryls present in the viral coat proteins of the recombinant virus to the primary amines present on the targeting element.

1. *Reduction of GDV Proteins to Create Free Sulfhydryls*

This procedure may be used when the GDV proteins have insufficient free sulfhydryl groups required for conjugation. A GDV solution is prepared to an equivalent protein concentration of 4.0 mg/mL in a phosphate buffered saline (PBS) solution containing EDTA to a final concentration of 5 mM. A reducing agent is added to this solution containing 0.5 M b-2-mercaptoethanolamine in PBS with 5.0 mM EDTA and the reaction mixture is incubated for 90 minutes at 37°C. Following incubation the modified GDV may be desalted by column chromatography.

2. *Maleimide Activation of the Targeting element*

Approximately 1.0 mg of the linker in 50 μL dimethylsulfoxide (DMSO) is added to 4.0 mg/mL of targeting element in 500 μL PBS buffer, pH 7.2. The reaction mixture is incubated for 60 minutes at room temperature. The maleimide activated-targeting element may be desalted by column chromatography.

3. *Conjugation*

The reduced GDV is mixed with the maleimide activated-targeting element and incubated at 4°C overnight. The targeting element conjugated GDV may be desalted by column chromatography.

C. Conjugation using Sulfosuccinimidyl (4(azidosalicylamido)hexanoate

Sulfosuccinimidyl (4(azidosalicylamido)hexanoate (sulfo-NHS-LC-ASA, Pierce, Rockford, IL) is photoreactive, consequently some parts of the following conjugation procedure must be performed in a darkened room. Approximately 3 mg of sulfo-NHS-LC-ASA is dissolved in 50 μL of DMSO. This stock solution is diluted 1:200 with 0.1 M sodium phosphate buffer, pH 7.4. A 2 to 50 molar excess of the linker is added to 4.0 mg/mL of targeting element in 500 μL PBS buffer, pH 7.2. The reaction mixture is incubated for 60 minutes at room temperature. The maleimide activated-targeting element may be desalted by column chromatography. An equivalent of 1.0 mg of the GDV dissolved in 500 μL PBS is then added to this mixture. The reaction mixture is incubated for 15 minutes at 37°C and then irradiated with long wave UV light for 10 minutes at room temperature. This mixture is then flashed with a bright light for 1 to 3 seconds (three camera flashes). The photoactivated targeting element conjugated GDV may be desalted by column chromatography.

D. Conjugation using Sulfosuccinimidyl-2-[6-(biotinamido)-2-(p-azidobenzamido)-hexanoamido]ethyl-1,3'-dithiopropionate

Sulfosuccinimidyl-2-[6-(biotinamido)-2-(p-azidobenzamido)-hexanoamido]ethyl-1,3'-dithiopropionate (Sulfo-SBED, Pierce, Rockford, IL) is a trifunctional crosslinking reagent having biotin covalently attached to a heterobifunctional reagent comprising a

hydroxysuccinimido active ester and a photoreactive aryl azide. Approximately 1.12 mg of Sulfo-SBED is dissolved in 25 μL DMSO. Approximately 11 μL of the Sulfo-SBED is then added to a solution of targeting element containing 5 mg of the targeting element in 0.5 mL of 0.1 M PBS, pH 7.2. This mixture is incubated at room temperature for 30 minutes. The linking agent-targeting element conjugate may be desalted by column chromatography. The linking agent-targeting element conjugate is then mixed with the GDV solution having an equivalent protein concentration of 5.0 mg dissolved in 0.5 mL PBS and incubated at room temperature for 3.5 minutes. This reaction mixture is irradiated with long wave UV light for 15 minutes. The targeting element-GDV conjugate may be desalted by column chromatography. Since the linking agent is biotinylated a second molecule conjugated to avidin may be bound to this targeting element-GDV conjugate.

E. Conjugation using Disuccinimidyl Suberate

Disuccinimidyl suberate (DSS, Pierce, Rockford, IL) is a homobifunctional N-hydroxysuccinimdyl ester linking agent. This protocol provides for the conjugation of a GDV to a targeting element via primary amines present on the proteins of the GDV and the targeting element. A protein concentration equivalent of 0.1 to 0.5 mg of the GDV in PBS is incubated with the targeting element having a concentration of 5 to 10 nM in PBS in a total volume of 100 μL for 1.0 hour at 4°C. To this mixture, DSS solution (DSS dissolved in dry DMSO to a 10-25 mM concentration) is added to a final concentration of 1 to 2 mM and allowed to react for 30 minutes to 2 hours. Following incubation a stop solution (1.0 M Tris, pH 7.5) is added to a final concentration of 10 to 20 mM and the reaction mixture is incubated for 15 minutes. The targeting element-GDV may be desalted by column chromatography.

F. Conjugation of MSH to GDV Utilizing a Carbohydrate Linker

Incubate 0.1 to 0.5 mg of melanocyte stimulating hormone (Chiron Mimotopes, San Diego, CA) in 5 to 10 mM glucosamine (Sigma, St. Louis, MO) in a total volume of 100 μL for 1.0 hours at 4°C. DSS solution (DSS dissolved in dry DMSO to this mixture to a 10-25 mM concentration) is added to a final concentration of 1 to 2 mM and incubated for 30 minutes to 2 hours. Following incubation a stop solution (1.0 M Tris, pH 7.5) is added to a

final concentration of 10 to 20 mM and the reaction mixture is incubated for 15 minutes. The MSH-glucose conjugate is added to a cold solution of sodium *meta*-periodate containing 100 mM sodium periodate in 0.1 M sodium acetate buffer, pH 5.5. The oxidation reaction is allowed to proceed for 1.0 hour in the dark at room temperature. Glycerol is added and the mixture is dialyzed at 4°C overnight and then concentrated.

G. Coupling IgG to Carboxylated PE Liposome Suspensions

A liposomal suspension (1.5 μmole) is mixed with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC; 4 mg, Pierce Chemical Co., Rockford, IL) in 1.5 mL of 10 mM $NaPO_4$, 0.15 M NaCl, pH 5.0. The reaction is carried out at room temperature for one hour.

The liposome/EDC mixture (1.5 mL) is mixed with 75 μL of mouse IgG (Cappel Labs, Malvern, PA; 10 mg/mL) and 75 μL of 1 M NaCl, and the coupling-reaction mixture adjusted to pH 8.0. Each reaction is carried out overnight at 4°C. Unreacted protein is separated from liposome-conjugated protein by metrizamide density gradient centrifugation, according to a standard procedure. Control coupling reactions are performed by substituting buffer for EDC.

The amount of protein bound to the liposome is determined by the Lowry protein assay. The concentration of lipid is determined from I^{125} radioactivity levels, based on a known amount of PE-I^{125} included in the liposome preparations. Based on the measured protein and lipid concentrations, the protein to lipid coupling ratios, expressed in micrograms protein/μmole, lipid concentrations are determined.

Example 2

ASSAYS FOR GDV UPTAKE AND FUNCTIONAL GENE EXPRESSION

A. Assay for GDV Uptake

Human hepatoma cell lines HepG2 (Schwartz, *et al.*, *J. Biol. Chem. 256*:8878, 1981) and SK Hep 1, and rat hepatoma cell line Morris 7777 (ATCC CRL 1601, Wu *et. al.*, *J. Biol. Chem. 263*:4719, 1988) and murine fibroblast cell line NIH3T3 (ATCC CRL 1658, Goud *et al.*, *Vir. 163*:251, 1988) are plated at a density of 0.5 to 2.0 x 10^5 cells/mL in 60

mm plastic dishes (Falcon Scientific Co., Lincoln Park, NJ). Equal amounts (16.7 μg of RNA, 0.5 mg of viral protein) of modified and unmodified GDV in Dulbecco's modified Eagle's medium are added to the culture medium and exposed to cells for 5 days at 37°C under 5% CO_2. Cells are assayed for b-galactosidase activity as a measure of foreign gene expression according to the method of Gorman (*DNA Cloning 2*:157-158, 1986, Glover, D.M., ed., IRL Press, Washington, DC). In brief, cell monolayers (approximately 1.0 x 10^6 cells/60 mm dish) are washed with phosphate-buffered saline, then lysed. The lysate, 0.1 mL, is reacted with *o*-nitrophenyl galactopyranoside (Sigma, St. Louis, MO) and b-galactosidase activity quantitated by absorbance at 420 nm after the addition of Na_2CO_3 to terminate the reaction.

B. Histochemical Staining to Demonstrate b-Galactosidase Activity

To determine the fraction of cells that expressed the b-galactosidase gene after exposure to targeted GDV samples, histochemical staining of *in situ* b-galactosidase activity is performed according to the method of Sanes, *et al*. (*EMBO J. 5*:3133, 1986). In brief, cultured cells in 35 mm dishes containing 0.5 to 1.0 x 10^6 cells are treated for 5 days with equal amounts (8.4 μg of viral RNA, 0.3 mg of viral protein) of modified or unmodified virus. Cells are fixed in 0.5% glutaraldehyde (Sigma, St. Louis, MO), phosphate-buffered saline, then incubated with 1.0 mM $MgCl_2$ phosphate-buffered saline, and overlaid with 1.0 mg/mL X-gal (GIBCO, Bethesda Research Laboratories, MD), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$ in phosphate-buffered saline. After incubation at 37°C for 1 hour, the dishes are washed in phosphate-buffered saline to quench the reaction and evaluated by counting positive (blue) cells under a light microscope, and the results are expressed as the percent of positive 10 high power fields.

C. Assays for Cellular Uptake of GDV

To determine whether the targeted GDV according to the invention is taken up by cells, the cells may be incubated at 37°C in serum-free Dulbecco's modified Eagle's medium containing ^{35}S-biolabeled, modified GDV, 3.3 μg of viral RNA (98 μg of viral protein) (Watanabe, *et al., Cancer Immunol. Immunother. 28*:157, 1989) with a specific activity of

6.1 x 10^5 cpm/mg of nucleic acid. At various times, medium is removed, and cells are chilled to 4°C and washed with ice-cold minimum essential medium containing 1.0 mg/mL bovine serum albumin. Surface-bound radioactivity is stripped with 0.5 mL of cold phosphate-buffered saline, pH 7.2, containing 0.4% trypsin, 0.02% EDTA, and separated from cells by centrifugation. The cell pellet is solubilized in 0.2 N NaOH and Poly-Fluor (Packard Instrument Co.), and trypsin-EDTA-resistant (internalized) radioactivity is measured by scintillation counting (Tr-Carb 4530, Packard) (Schwartz, *et al., J. Biol. Chem. 256*:8878, 1981). Nonspecific uptake is measured in the presence of a 100-fold molar excess of targeting element, and specific uptake is calculated as the difference between total and nonspecific measurements.

D. Stability of Modified GDV

To assess the stability of the modified GDV, samples of freshly prepared sterile, GDV conjugated to the desired targeting element are incubated in serum-free Dulbecco's modified Eagle's medium at 4 and 25°C. At various times, samples are added to the medium of target cells and incubated for 5 days. Cells are then assayed for b-galactosidase activity by colorimetric assay as described above.

E. Determination of Protein Expression by ELISA

Cell lysates from cells transduced by SK+HBe-c are made by washing 1.0 x 10^7 cultured cells with PBS, resuspending the cells in a total volume of 600 ml on PBS, and sonicating for two 5-second periods at a setting of 30 in a Branson sonicator, Model 350, (Fisher, Pittsburgh, PA) or by freeze thawing three times. Lysates are clarified by centrifugation at 10,000 rpm for 5 minutes.

Core antigen and precore antigen in cell lysates and secreted e antigen in culture supernatant are assayed using the Abbott HBe, rDNA EIA kit (Abbott Laboratories Diagnostic Division, Chicago, IL). Another sensitive EIA assay for precore antigen in cell lysates and secreted e antigen in culture supernatant is performed using the Incstar ETI-EB kit, (Incstar Corporation, Stillwater, MN). A standard curve is generated from dilutions of recombinant hepatitis B core and e antigen obtained from Biogen (Geneva, Switzerland).

Using these procedures, approximately 20-40 ng/ml HBV e antigen is expressed in transduced cell lines, and 38-750 ng/ml of HBV core antigen is expressed in transduced cell lines. Alternatively, protein expression may be determined by Western blot or by Immunoprecipitation/Western blot. *See* U.S.S.N. 08/483,511, filed June 7, 1995.

F. Determination of Protein Expression by Luminescence

When the vector expresses the luciferase marker, expression may be assayed by exposing the sample to luciferin and measuring the resulting luminescence. Briefly, transfected cells are harvested, washed in PBS and resuspended in 200 mL of 0.25 M Tris-HCl, pH 7.8. The cells are lysed by three cycles of freeze/thawing and the cellular debris is removed by centrifugation. Approximately 50 mL of cell lysate is assayed for luciferase activity by measuring light emission with a bioluminometer (Analytical Bioluminescence, San Diego, CA) in the presence of luciferin and ATP (Brasier *et al.*, *Biotechniques* 7:1116, 1989). The amount of protein in the lysate is determined by the Bradford dye-binding procedure (Bio-Rad, Hercules, CA).

Example 3

ADMINISTRATION PROTOCOLS

A. Administration to Animals Other than Humans

Targeted GDV preparations made in accordance with the teachings provided herein are injected into an animal at doses of 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, or 10^{11} GDVs with or without uptake enhancers such as polybrene (1-8 μg/mL) or DEAE dextran (2 - 30 μg/mL). Injections are given daily for 1, 2, 3, 4, 5, 6, or 7 days, and 2 to 7 days after the last injection, to determine the activity of the delivered gene. Injections are typically administered through an I.V.

B. Administration to Humans

Patients preferably receive doses of about 10^6, 10^7, 10^8, 10^9, 10^{10}, or 10^{11} targeted GDVs I.V., intra-arterially, in the local vasculature or peritumorally, as the case may be, in a volume of 0.1 to 3 mL. If the gene is one that encodes a protein which converts a non-toxic

precursor (prodrug) into a toxic product , the prodrug is administered at doses defined in the Physicians Desk Reference or those predicted from animal experiments at times of between 1 to 30 days after the last administration of the targeted GDV.

The targeted GDV is typically administered from 1 to 20 times at intervals of 1 to 15 days and the patient status is monitored by following normal clinical parameters and monitoring tumor sizes by radiography, MRI scans, PET scans or other conventional means.

While the present invention has been described above both generally and in terms of preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art in light of the description, *supra.* Therefore, it is intended that the appended claims cover all such variations coming within the scope of the invention as claimed.

Additionally, the publications and other materials cited to illuminate the background of the invention, and in particular, to provide additional details concerning its practice as described in the detailed description and examples, are hereby incorporated by reference in their entirety.

In the Claims:

1. A gene delivery vehicle to which a targeting element is covalently bound by a linking agent wherein the targeting element is capable of interacting with a molecule present on the surface of a target cell.

2. A gene delivery vehicle according to claim 1, wherein the linking agent is selected from the group consisting of a homobifunctional linking agent, a heterobifunctional linking agent, and a trifunctional linking agent.

3. A gene delivery vehicle according to claim 2, wherein the linking agent is selected from the group consisting of 4(4-N-maleimidophenyl)butyric acid hydride•HCl•1/2 dioxane (MPBH), succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), sulfosuccinimidyl (4(azidosalicylamido)hexanoate (Sulfo-NHS-LC-ASA), sulfosuccinimidyl -2- [6-(biotinamido) -2- (*p*-azidobenzamido)-hexanoamido] ethyl-1,3'-dithiopropionate (Sulfo-SBED), and disuccinimidyl suberate (DSS), N-succinimidyl-3-(2-pyridyldithio) - propionate (SPDP)), sulfo - succinimidyl 4 - (N-maleimidomethyl) cyclohexane - 1 - carboxylate (Sulfo-SMCC), 1 - ethyl - 3 - (3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), and bis-diazobenzidine (BDB).

4. A gene delivery vehicle according to claim 1, wherein the linking agent comprises a carbohydrate linking agent.

5. A gene delivery vehicle according to claim 4, wherein the carbohydrate of the carbohydrate linking agent is selected from the group consisting of a monosaccharide, a disaccharide, and an oligosaccharide.

6. A gene delivery vehicle according to claim 5, wherein the carbohydrate of the carbohydrate linking agent is a monosaccharide linking agent selected from the group consisting of fructosamine, glucosamine, galactosamine, and mannosamine.

7. A gene delivery vehicle according to claim 5, wherein the carbohydrate of the carbohydrate linking agent is a disaccharide linking agent selected from the group consisting of aminated sucrose, aminated maltose, aminated trehalose, and aminated lactose, or a oligosaccharide linking agent selected from the group consisting of N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, aminated N-acetylmuramic acid, and aminated N-acetyl-D-neuraminic acid.

8. A target cell transduced with a gene delivery vehicle according to claims 1-7.

9. A pharmaceutical composition comprising a gene delivery vehicle according to claima 1-7 in a pharmaceutically acceptable carrier.

10. A method of preparing a gene delivery vehicle according to claims 1-7 comprising mixing a gene delivery vehicle and a targeting element in the presence of a linking agent under conditions where the linking agent becomes covalently linked to both the gene delivery vehicle and targeting element.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/20543

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/00 A61K48/00 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIOCONJUGATE CHEMISTRY, vol. 2, no. 4, 1 January 1991, pages 226-231, XP002002761 WAGNER E ET AL: "DNA-BINDING TRANSFERRIN CONJUGATES AS FUNCTIONAL GENE-DELIVERY AGENTS: SYNTHESIS BY LINKAGE OF POLYLYSINE OR ETHIDIUM HOMODIMER TO THE TRANSFERRIN CARBOHYDRATE MOIETY" see figure 1 see paragraph Discussion --- -/--	1-10

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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2

Date of the actual completion of the international search	Date of mailing of the international search report
15 May 1997	05.06.97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Dullaart, A

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 88, October 1991, pages 8850-8854, XP002001452 CURIEL D T ET AL: "ADENOVIRUS ENHANCEMENT OF TRANSFERRIN-POLYLYSINE-MEDIATED GENE DELIVERY" see paragraph materials and methods	1-10
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 89, no. 17, 1 September 1992, pages 7934-7938, XP000371760 WAGNER E ET AL: "INFLUENZA VIRUS HEMAGGLUTININ HA-2 N-TERMINAL FUSOGENIC PEPTIDES AUGMENT GENE TRANSFER BY TRANSFERRIN-POLYLYSINE-DNA COMPLEXES: TOWARD A SYNTHETIC VIRUS-LIKE GENE-TRANSFER VEHICLE" see abstract see paragraph Discussion	1-10
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 87, 1 May 1990, pages 3410-3414, XP002002759 WAGNER E ET AL: "TRANSFERRIN-POLYCATION CONJUGATED AS CARRIERS FOR DNA UPTAKE INTO CELLS" see paragraph materials and methods	1-10
X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 264, no. 29, 15 October 1989, US, pages 16985-16987, XP002030967 C.H. WU ET AL.: "Targeting genes: delivery and persistent expression of a foreign gene driven by mammalian regulatory elements in vivo" see page 16985, right-hand column	1-10
X	DE 41 15 038 A (GENENTECH INC ;BOEHRINGER INGELHEIM INT (DE)) 12 November 1992 see abstract see figure 1; example 1 see claims	1-10
X	WO 94 10323 A (IMPERIAL CANCER RESEARCH TECHMOLOGY LTD) 11 May 1994 see abstract see table 2	1-10
X	WO 94 25608 A (BAYLOR COLLEGE MEDICINE) 10 November 1994 see examples	1-10

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/20543

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 31566 A (VIAGENE INC) 23 November 1995 see abstract see examples see claims	1-10
X,P	WO 96 15811 A (IMPERIAL COLLEGE ;HART STEPHEN LEWIS (GB); HARBOTTLE RICHARD PAUL) 30 May 1996 see examples	1-10

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 96/ 20543

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [X] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

Claims searched incompletely: 1-7, 9, 10
Claims not searched: 8

See next page.

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

In view of the large number of compounds which are defined by the general definition used in the independent claims, the search had to be restricted for economic reasons. The search was limited to the compounds for which pharmacological data was given and/or the compounds mentioned in the claims and to the general idea underlying the applicattion (see Guidelines, chapter III, paragraph 2.3). There is no indication in the present application how a cell transduced with a gene delivery vehicle according to the present application can be distinguished from a cell, transduced with a different technique.

INTERNATIONAL SEARCH REPORT

Information on patent family members

ernational Application No
PCT/US 96/20543

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE 4115038 A	12-11-92	CA 2105771 A	09-11-92
		WO 9219281 A	12-11-92
		EP 0584118 A	02-03-94
		JP 6507158 T	11-08-94
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WO 9531566 A	23-11-95	AU 2589695 A	05-12-95
		CA 2158926 A	14-11-95
		EP 0759087 A	26-02-97
WO 9615811 A	30-05-96	AU 3876695 A	17-06-96